# N-Terminal PVDF 4.0 Protein Sequencer Methods

## Technical Note

**HP 241 Protein Sequencer (N+C)**

The N-terminal PVDF 4.0 sequencer methods implement the Edman degradation chemistry (coupling, cleavage, and conversion) on the Hewlett-Packard column-based protein sequencer. Reagents 1R, 2R, 3R, and the TH-Std are specific for C-terminal sequencing and are not utilized for the N-terminal PVDF 4.0 sequencer methods.

These methods are suitable for N-terminal sequence analysis of most protein and peptide samples in the low nanomole to low picomole range which have been loaded onto a Hewlett-Packard PVDF column. The column should be pretreated with the Column prep 4.0 method prior to sample loading. For protein samples, the PVDF column consists of an empty SAX column on top and a RP column on the bottom. For peptide samples, the PVDF column consists of an empty RP column on top and a SAX column on the bottom. The PVDF strip is placed in the empty top column.

The methods are:
- N-Terminal PVDF 4.0
- N-Terminal PVDF 4.0 (Cycle 1)
- N-Terminal Flask 4.0
- PTH-Std 4.0
- Column Prep 4.0

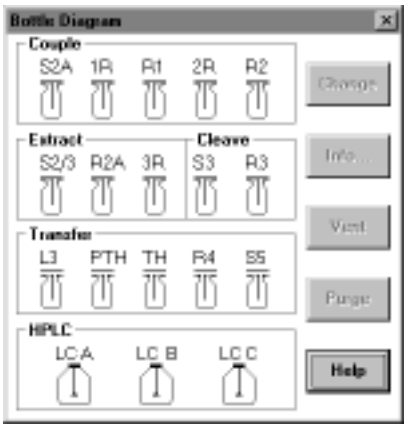
The column and flask methods control all of the derivatization/cleavage and conversion reactions, respectively.

The sequence program controls all of the column and flask methods, and well as any cycle exception methods. The N-terminal PVDF 4.0 sequencer method uses a cycle 1 column exception method.

The cycle 1 method provides a longer initial drying time and a pause to provide time for proper HPLC column equilibration after switching from C-terminal sequencing. This method also performs a double coupling on the N-terminal amino acid. There is no specialized cycle 1 method for the flask.

The PTH Std 4.0 method delivers 10 pmol/100 µl PTH-amino acid standard solution to the on-line HPLC allowing the quantitation and identification of sequenced amino acids. One may choose to run a PTH-standard by using PTH-std 4.0 when scheduling a sample.

The column prep 4.0 method is used to wash the sequencer column prior to sample loading. This has been found to be particularly important for low-level sample quantities (<10 pmol) samples.




The Routine 4.0 sequencer methods require the bottle configuration shown. The reagents and solvents are purchased from Hewlett-Packard. These methods produce less than 1mL/cycle of liquid waste.

| | |
|---|---|
| **S2A** | Neat ethyl acetate |
| **1R** | Diphenylphosphorylisothiocyanate (DPP-ITC) in toluene/heptane (23:27:50) |
| **R1** | Phenylisothiocyanate (PITC) in heptane (3:97) |
| **2R** | Pyridine in ethylacetate (2:98) |
| **R2** | Diisopropylethylamine (DIEA) in 1-propanol/water (1:3:6) |
| **S2/3** | Acetonitrile/toluene (23:77) |
| **R2A** | Ocylamine in heptane (3:97) |
| **3R** | Potassium trimethylsilanolate (0.1M) in methanol/t-butanol (50:50) |
| **S3** | Acetonitrile/toluene (15:85) |
| **R3** | Neat trifluoroacetic acid (TFA) |
| **L3** | Acetic acid in methanol/water (1:74:25) |
| **PTH** | Mixture of PTH amino acids (10 pmol/100 µl) in acetonitrile with DPTU as a marker |
| **TH** | Mixture of TH amino acids (50 pmol/100 µl) in acetonitrile |
| **R4** | Trifluoroacetic acid (TFA) in water (1:3) |
| **S5** | Phosphate buffer (pH 2.9), 0.3% ion pairing reagent |

**Method Details**

The steps for the N-terminal PVDF 4.0 column methods are described below. The steps indicate the ranges in volume that are appropriate for the various reagents/solvent deliveries. All sequencer methods are accessed by choosing the top menu item Edit/Method in the Protein Sequencer window. There are two sequencer column configurations that can be used with the N-terminal PVDF 4.0 methods. For protein samples, the PVDF column consists of an empty SAX column on top and a RP column on the bottom. For peptide samples, the PVDF column consists of an empty RP column on top and a SAX column on the bottom. The PVDF strip is placed in the empty top column. The column should be pre-treated with the Column prep 4.0 method prior to sample loading.

**Method (Column): N-Terminal PVDF 4.0**

Total time: 45.7 min

All metering steps deliver the specified reagent or solvent to waste.

**Step 3:** The delivery of R2A should completely wet the membrane as well as the bottom column.

**Steps 6, 9:** The delivery of R1 should completely wet the membrane and only slightly wet the top of the bottom column. The second R1 delivery may require a shorter meter time since the R1 bottle was already pressurized for the first delivery.

**Step 12:** The volume of R2 delivered by the end of Step 12 should completely wet the membrane and only slightly wet the top of the bottom column.

**Step 14:** "Flush with S2A" flushes the delivery lines and should not wet the column

**Steps 19, 21, 23, 25, 34, 38:** The delivery of S2A should completely fill the column, followed by drying.

**Step 29:** During Step 29, no more than the upper half of the membrane should be wet with R3. The TFA should wick down to wet the whole membrane after delivery. The bottom column should not be wet.

**Step 32:** After step 32, the volume of L3 delivered to the flask should be 50 µl (+/- 5 µl)

| Step | Description | Primary Time | Temperature |
|---|---|---|---|
| 1: | Couple: Dry column DOWN | 60.0 | 55 |
| 2: | Couple: Meter R2A | 5.2 | 55 |
| 3: | Couple: Deliver R2A DOWN (closed) | 20.0 | 55 |
| 4: | Couple: Dry column DOWN | 60.0 | 55 |
| 5: | Couple: Meter R1 | 2.5 | 55 |
| 6: | Couple: Deliver R1 DOWN (closed) | 20.0 | 55 |
| 7: | Couple: Dry column DOWN | 60.0 | 55 |
| 8: | Couple: Meter R1 | 2.5 | 55 |
| 9: | Couple: Deliver R1 DOWN (closed) | 20.0 | 55 |
| 10: | Couple: Dry column DOWN | 120.0 | 55 |
| 11: | Couple: Meter R2 | 11.0 | 55 |
| 12: | Couple: Deliver R2 DOWN | 20.0 | 55 |
| 13: | Couple: Dry column DOWN | 60.0 | 55 |
| 14: | Couple: Flush with S2A | 15.0 | 55 |
| 15: | Couple: React | 300.0 | 55 |
| 16: | Couple: Deliver from coupling bank DOWN | 60.0 | 55 |
| 17: | Couple: Dry column UP | 120.0 | 55 |
| 18: | Wash: Meter S2A | 14.0 | 50 |
| 19: | Wash: Deliver and DRY DOWN (closed) | 30.0 | 50 |
| 20: | Wash: Meter S2A | 14.0 | 50 |
| 21: | Wash: Deliver and DRY DOWN (closed) | 30.0 | 50 |
| 22: | Wash: Meter S2A | 14.0 | 50 |
| 23: | Wash: Deliver and DRY DOWN (closed) | 30.0 | 50 |
| 24: | Wash: Meter S2A | 14.0 | 50 |
| 25: | Wash: Deliver and DRY DOWN (closed) | 30.0 | 50 |
| 26: | Wash: Dry column DOWN | 150.0 | 50 |
| 27: | Cleave: Purge cleavage line | | 50 |
| 28: | Cleave: Meter R3 | 2.9 | 50 |
| 29: | Cleave: Deliver R3 DOWN | 30.0 | 50 |
| 30: | Cleave: React | 350.0 | 50 |
| 31: | !Cleave: Dry column DOWN to flask | 60.0 | 50 |
| 32: | !Extract: Deliver L3 to flask | 7.0 | 50 |
| 33: | !Extract: Meter S2A | 14.0 | 50 |
| 34: | !Extract: Deliver solvent DOWN (closed) | 15.0 | 50 |
| 35: | !Extract: Dry column DOWN to flask | 5.0 | 50 |
| 36: | !Extract: Evaporate flask | 50.0 | 50 |
| 37: | !Extract: Meter S2A | 14.0 | 50 |
| 38: | !Extract: Deliver solvent DOWN (closed) | 15.0 | 50 |
| 39: | !Extract: Dry column DOWN to flask | 5.0 | 50 |
| 40: | !Extract: Evaporate flask | 20.0 | 50 |
| 41: | Extract: Dry column DOWN to waste | 60.0 | 55 |
| 42: | Couple:Dry column UP | 60.0 | 55 |

**Method (Column): N-Terminal PVDF 4.0 (Cycle 1)**

Total time: 76.2 min

**Step 1:** Cycle 1 begins with an extended dry to remove any residual solvent from the sample loading process. There is also a pause to provide time for proper HPLC column equilibration after switching from C-terminal sequencing.

**Step 3:** The delivery of R2A should completely wet the membrane as well as the bottom column.

**Steps 6, 9, 18:** The delivery of R1 should completely wet the membrane and only slightly wet the top of the bottom column. The second and third R1 delivery may require a shorter meter time since the R1 bottle was already pressurized for the first delivery.

**Step 12, 21:** The volume of R2 delivered by the end of Step 12 and 21 should completely wet the membrane and only slightly wet the top of the bottom column. The second R2 delivery may require a shorter meter time since the R2 bottle was already pressurized for the first delivery.

**Step 23, 50:** "Flush with S2A" flushes the delivery lines and should not wet the column

**Steps 28, 30, 32, 34, 43, 47:** These delivery of S2A should completely fill the column, followed by drying.

**Step 38:** During Step 38, no more than the upper half of the membrane should be wet with R3. The TFA should wick down to wet the whole membrane after delivery. The bottom column should not be wet.

**Step 41:** After step 41, the volume of L3 delivered to the flask should be 50 µl (+/- 5 µl)

| Step | Description | Primary Time | Temperature |
|---|---|---|---|
| 1: | Couple: Dry column DOWN | 300.0 | 55 |
| 2: | Couple: Meter R2A | 5.2 | 55 |
| 3: | Couple: Deliver R2A DOWN (closed) | 20.0 | 55 |
| 4: | Couple: Dry column DOWN | 60.0 | 55 |
| 5: | Couple: Meter R1 | 2.5 | 55 |
| 6: | Couple: Deliver R1 DOWN (closed) | 20.0 | 55 |
| 7: | Couple: Dry column DOWN | 60.0 | 55 |
| 8: | Couple: Meter R1 | 2.5 | 55 |
| 9: | Couple: Deliver R1 DOWN (closed) | 20.0 | 55 |
| 10: | Couple: Dry column DOWN | 120.0 | 55 |
| 11: | Couple: Meter R2 | 11.0 | 55 |
| 12: | Couple: Deliver R2 DOWN | 20.0 | 55 |
| 13: | Couple: Dry column DOWN | 60.0 | 55 |
| 14: | Couple: React | 300.0 | 55 |
| 15: | Couple: Deliver from coupling bank DOWN | 60.0 | 55 |
| 16: | Couple: Dry column DOWN | 60.0 | 55 |
| 17: | Couple: Meter R1 | 2.5 | 55 |
| 18: | Couple: Deliver R1 DOWN (closed) | 20.0 | 55 |
| 19: | Couple: Dry column DOWN | 60.0 | 55 |
| 20: | Couple: Meter R2 | 11.0 | 55 |
| 21: | Couple: Deliver R2 DOWN | 20.0 | 55 |
| 22: | Couple: Dry column DOWN | 60.0 | 55 |
| 23: | Couple: Flush with S2A | 15.0 | 55 |
| 24: | Couple: React | 300.0 | 55 |
| 25: | Couple: Deliver from coupling bank DOWN | 60.0 | 55 |
| 26: | Couple: Dry column UP | 120.0 | 55 |
| 27: | Wash: Meter S2A | 14.0 | 50 |
| 28: | Wash: Deliver and DRY DOWN (closed) | 30.0 | 50 |
| 29: | Wash: Meter S2A | 14.0 | 50 |
| 30: | Wash: Deliver and DRY DOWN (closed) | 30.0 | 50 |
| 31: | Wash: Meter S2A | 14.0 | 50 |
| 32: | Wash: Deliver and DRY DOWN (closed) | 30.0 | 50 |
| 33: | Wash: Meter S2A | 14.0 | 50 |
| 34: | Wash: Deliver and DRY DOWN (closed) | 30.0 | 50 |
| 35: | Wash: Dry column DOWN | 150.0 | 50 |
| 36: | Wash: Purge solvent line | | 50 |
| 37: | Cleave: Meter R3 | 2.9 | 50 |
| 38: | Cleave: Deliver R3 DOWN | 30.0 | 50 |
| 39: | Cleave: React | 350.0 | 50 |
| 40: | !Cleave: Dry column DOWN to flask | 60.0 | 50 |
| 41: | !Extract: Deliver L3 to flask | 7.0 | 50 |
| 42: | !Extract: Meter S2A | 14.0 | 50 |
| 43: | !Extract: Deliver solvent DOWN (closed) | 15.0 | 50 |
| 44: | !Extract: Dry column DOWN to flask | 5.0 | 50 |
| 45: | !Extract: Evaporate flask | 50.0 | 50 |
| 46: | !Extract: Meter S2A1 | 4.0 | 50 |
| 47: | !Extract: Deliver solvent DOWN (closed) | 15.0 | 50 |
| 48: | !Extract: Dry column DOWN to flask | 5.0 | 50 |
| 49: | !Extract: Evaporate flask | 20.0 | 50 |
| 50: | Wash: Flush with S2A | 15.0 | 55 |
| 51: | Extract: Dry column DOWN to waste | 60.0 | 55 |
| 52: | Couple: Dry column UP | 60.0 | 55 |

**Method (Flask): N-Terminal Flask 4.0**

Total time: 34.6 min

**Step 1:** During Step 1, the liquid in the flask partially evaporates but must not dry down completely (10 - 20 µl remains). The flask will continue to dry during the first part of Step 2.

**Step 2:** During Step 2, the volume of R4 delivered to the flask should be approximately 70 µl (+/- 5 µl)

**Step 4:** By the end of Step 4, the flask will have been dry for 100-150 seconds.

**Step 5:** By the end of Step 5, 70 µl (+/- 5 µl) of L3 will have been delivered to the flask

**Step 6:** By the end of Step 6, the flask will have been dry for 75-100 seconds

**Step 7:** The delivery of L3 may not be visible

**Step 8:** After Step 8, the volume of L3 and S5 in the flask should be 75-80 µl. If adjustment is needed, it should be done by adjusting the metering time of S5, leaving L3 unchanged.

***TEST Delivery protocol:***
*To check the delivery volume of L3 and S5, copy steps 7, 8, 9, and 10 to the clipboard and run from the clipboard. At the end of Step 10, remove the flask from the heating chamber. Carefully unscrew the flask cap and measure the delivered volume using a syringe. Be careful that some of the delivered volume is not retained on the tube sides or top of the flask.*

**Step 11:** Before injection the injector loop should be filled, leaving the solvent visible in both the inlet and outlet lines of the injector loop. The time for Step 11 may have to be adjusted in order to optimize the delivery.

| Step | Description | Primary Time | Temperature |
|---|---|---|---|
| 1: | Convert: Evaporate | 40.0 | 70 |
| 2: | Convert: Deliver R4 | 29.0 | 70 |
| 3: | Convert: React | 400.0 | 70 |
| 4: | Convert: Evaporate dry | 400.0 | 70 |
| 5: | Convert: Flush with L3 | 15.0 | 70 |
| 6: | Convert: Evaporate dry L3 | 140.0 | 70 |
| 7: | Convert: Deliver L3 dripwise | 2.0 | 60 |
| 8: | Convert: Deliver S5 dripwise | 12.0 | 60 |
| 9: | Convert: Mix and equilibrate | 15.0 | 60 |
| 10: | Convert: Solubilize | 20.0 | 60 |
| 11: | Convert: Fill loop and inject | 2.0 | 60 |
| 12: | Convert: Empty flask | 30.0 | 60 |
| 13: | Convert: Flush with L3 | 85.0 | 45 |
| 14: | Convert: Mix | 20.0 | 45 |
| 15: | Convert: Empty flask | 30.0 | 45 |
| 16: | Convert: Deliver R4 wash | 40.0 | 45 |
| 17: | Convert: Empty flask | 30.0 | 45 |
| 18: | Convert: Deliver R4 wash | 40.0 | 45 |
| 19: | Convert: Empty flask | 30.0 | 45 |
| 20: | Convert: Dry waste line | 60.0 | 45 |
| 21: | Convert: Dry vent line | 60.0 | 45 |

**Method (Flask): PTH Std 4.0**
Total time: 50.3 min

This PTH Std 4.0 method is appropriate with any of the N-terminal sequence programs. Ten picomoles of PTH standard are delivered to the flask in this method.

**Step 1: Step 1:** Cycle 1 begins with a pause to provide time for proper HPLC column equilibration after switching from C-terminal sequencing.

**Step 4:** Step 4 should add 100 µl (+/- 2 µl) of PTH-standard to the dry flask

***TEST Delivery protocol:***
*To check the delivery volume of the PTH-standards, copy the steps in the PTH Std Test 4.0 method to the clipboard and run from the clipboard. At the end of test program, remove the flask from the heating chamber. Carefully unscrew the flask cap and measure the delivered volume using a syringe. Be careful that some of the delivered volume is not retained on the tube sides or top of the flask*

**Step 6:** The delivery of L3 may not be visible

**Step 7:** After Step 7, the volume of L3 and S5 in the flask should be 75-80 µl. If adjustment is needed, it should be done by adjusting the metering time of S5, leaving L3 unchanged.

***TEST Delivery protocol:***
*To check the delivery volume of L3 and S5, copy steps 6, 7, 8, and 9 to the clipboard and run from the clipboard. At the end of Step 9, remove the flask from the heating chamber. Carefully unscrew the flask cap and measure the delivered volume using a syringe. Be careful that some of the delivered volume is not retained on the tube sides or top of the flask*

**Step 10:** Before injection the injector loop should be filled, leaving the solvent visible in both the inlet and outlet lines of the injector loop. The time for Step 10 may have to be adjusted in order to optimize the delivery.

| Step | Description | Primary Time | Temperature |
|---|---|---|---|
| 1: | Convert: Empty flask | 120.0 | 60 |
| 2: | Convert: Dry waste line | 200.0 | 60 |
| 3: | Convert: Dry vent line | 200.0 | 60 |
| 4: | Convert: Deliver PTH Std with L3 purge | 6.9 | 60 |
| 5: | Convert: Evaporate dry standard | 180.0 | 60 |
| 6: | Convert: Deliver L3 dripwise | 2.0 | 60 |
| 7: | Convert: Deliver S5 dripwise | 12.0 | 60 |
| 8: | Convert: Mix and equilibrate | 15.0 | 60 |
| 9: | Convert: Solubilize | 20.0 | 60 |
| 10: | Convert: Fill loop and inject | 2.0 | 60 |
| 11: | Convert: Empty flask | 30.0 | 60 |
| 12: | Convert: Flush with L3 | 120.0 | 45 |
| 13: | Convert: Mix | 20.0 | 45 |
| 14: | Convert: Empty flask | 30.0 | 45 |
| 15: | Convert: Deliver R4 wash | 40.0 | 45 |
| 16: | Convert: Empty flask | 30.0 | 45 |
| 17: | Convert: Deliver R4 wash | 40.0 | 45 |
| 18: | Convert: Empty flask | 30.0 | 45 |
| 19: | Convert: Dry waste line | 250.0 | 45 |
| 20: | Convert: Dry vent line | 250.0 | 45 |

**Method (Column): Column Prep 4.0**

Total time: 19.5 min

**Steps 3, 14, 20, 23, 25, 27, 29:** The delivery of S2A and S2/3 should completely fill the column, followed by drying.

**Steps 6, 17:** The delivery of R2A and R3 should completely fill the column (both halves)

**Step 9:** The delivery of R2 in Step 9 should completely fill the column (both halves)

| Step | Description | Primary Time | Temperature |
|---|---|---|---|
| 1: | Col prep: Purge coupling line | 8.0 | 60 |
| 2: | Col prep: Meter S2A | 12.0 | 60 |
| 3: | Col prep: Deliver from coupling bank DOWN | 15.0 | 60 |
| 4: | Col prep: Dry column DOWN | 10.0 | 60 |
| 5: | Col prep: Meter R2A | 10.0 | 60 |
| 6: | Col prep: Deliver R2A DOWN (closed) | 20.0 | 60 |
| 7: | Col prep: Dry column DOWN | 30.0 | 60 |
| 8: | Col prep: Meter R2 | 50.0 | 60 |
| 9: | Col prep: Deliver R2 DOWN (closed) | 45.0 | 60 |
| 10: | Col prep: wait | 90.0 | 60 |
| 11: | Col prep: Dry column DOWN to waste | 60.0 | 60 |
| 12: | Col prep: Purge coupling line | 30.0 | 60 |
| 13: | Col prep: Meter S2A | 12.0 | 60 |
| 14: | Col prep: Deliver from coupling bank DOWN | 15.0 | 60 |
| 15: | Col prep: Dry column DOWN | 120.0 | 60 |
| 16: | Col prep: Meter R3 | 15.0 | 60 |
| 17: | Col prep: Deliver from cleavage bank DOWN | | 60 |
| 18: | Col prep: Dry column DOWN | 10.0 | 60 |
| 19: | Col prep: Meter S2A | 12.0 | 60 |
| 20: | Col prep: Deliver from coupling bank DOWN | 15.0 | 60 |
| 21: | Col prep: Dry column DOWN | 60.0 | 60 |
| 22: | Col prep: Meter S2/3 | 17.0 | 60 |
| 23: | Col prep: Deliver and DRY DOWN (closed) | 30.0 | 60 |
| 24: | Col prep: Meter S2/3 | 17.0 | 60 |
| 25: | Col prep: Deliver and DRY DOWN (closed) | 30.0 | 60 |
| 26: | Col prep: Meter S2/3 | 17.0 | 60 |
| 27: | Col prep: Deliver and DRY DOWN (closed) | 30.0 | 60 |
| 28: | Col prep: Meter S2/3 | 17.0 | 60 |
| 29: | Col prep: Deliver and DRY DOWN (closed) | 30.0 | 60 |
| 30: | Col prep: Dry column DOWN | 60.0 | 60 |

**Hewlett-Packard shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance or use of this material.**

**Information, descriptions and specifications in this publication are subject to change without notice.**

**Copyright © 1997**
**Hewlett-Packard Company**
**All rights reserved. Reproduction and adaptation is prohibited.**

**Printed in the U.S.A. November 1997**
**5966-2774E**